HHS Public Access

Author manuscript

Cancer Epidemiol Biomarkers Prev. Author manuscript; available in PMC 2015 September 02.

Published in final edited form as:

Cancer Epidemiol Biomarkers Prev. 2012 January ; 21(1): 39–44. doi:10.1158/1055-9965.EPI-11-0800.

Development of a Method to Estimate Mouth-Level Benzo[*a*]pyrene Intake by Filter Analysis

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Abstract

Background—Benzo[*a*]pyrene (BaP) is one of the most potent carcinogens generated in cigarette smoke. During smoking, cigarette filters trap a significant portion of mainstream smoke benzo[a]pyrene. This trapped portion is proportional to what exits the end of the filter and is drawn into the mouth of smokers.

Methods—We developed a new method to estimate mouth-level BaP intake using filter analysis. In this analysis, cigarettes are smoked by a smoking machine using a variety of conditions to yield a range of mainstream smoke deliveries, which approximate a range of human puffing characteristics. Mainstream smoke BaP collected on Cambridge filter pads and the corresponding 1-cm mouth-end cigarette filter butts is extracted, purified by solid-phase extraction, and quantified by high-performance liquid chromatography coupled with a fluorescence detector. On the basis of the amount of BaP retained in cigarette butts and the amount collected on pads, we can relate them using a linear regression model.

Results—Using this model and subsequently analyzing cigarette filters collected from smokers, we are able to estimate their mouth-level intakes, which smokers received when they consumed cigarettes. We made a series of measurements using research cigarettes and select commercial cigarettes having a wide range of machine smoke "tar" and nicotine deliveries.

Conclusions—In all cases, results indicate a linear relation of BaP between cigarette filter butts and Cambridge filter pads, with R^2 ranging from 0.93 to 0.98.

Impact—This technique provides a noninvasive means to examine intake on a per cigarette basis to examine both exposure and behavioral aspects of smoking.

Introduction

A leading cause of lung cancer and related preventable diseases is tobacco smoke. Among the thousands of compounds generated in cigarette smoke, hundreds are identified as polycyclic aromatic hydrocarbons (PAH). One of the most commonly studied PAHs is benzo[a]pyrene; it is often used as a surrogate for other PAHs. Benzo[*a*]pyrene (BaP) was

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Disclosure of Potential Conflicts of Interest

recently reclassified as a group 1 carcinogen by International Agency for Research on Cancer based on sufficient evidence in animals and strong evidence that the mechanisms of carcinogenesis in animals also operate in exposed human beings (1). To estimate human exposure to benzo[a]pyrene, 2 approaches are commonly used, direct and indirect.

The direct approach involves detection of BaP in expired breath or its biomarkers in an appropriate human matrix (urine or serum). There is no evidence that BaP is eliminated via expired air after smoking (2). BaP is largely metabolized in the body prior to elimination. Less than 15% of BaP was found in mice or rats' urine after injection (2). In humans, metabolites of PAHs larger than pyrene were not detected in urine (3). BaP metabolism occurs in the liver; the primary route for hepatobiliary excretion is elimination in the feces. This matrix posts a challenge in method development for detecting metabolites of benzo[a]pyrene. Therefore, metabolites of pyrene, especially 1-hydroxypyrene, are commonly used as surrogate biomarkers to estimate human exposure to BaP (3).

The indirect approaches to estimating human exposure to BaP are typically much less invasive than collecting body fluids or excretion. One approach involves using specialized pressure transducers to record human smoking puff topography, that is, the duration and puff frequency. The puff topography data is then duplicated in machine smoking; mainstream smoke BaP is trapped on Cambridge filter pads (CFP). Assuming the smoker inhaled completely, the amount of BaP trapped in CFPs corresponds to the smoker's intake. Duplication of smoking behavior provides a pseudo–real-time estimate of the smoke puff volume that a smoker receives. In practice, such measurements are generally obtained in a laboratory setting using a cigarette holder, which can affect smoking behavior (4).

Another indirect approach is through discarded cigarette filter analysis. As smoke is drawn through the filter, a fraction is trapped before it exits the mouth end of the filter. The amount a smoker draws into his/her mouth is proportional to the trapped fraction (5). By measuring what is trapped in the filter, we can estimate the amount of mouth-level intake. This technique has been successfully used for more than 3 decades (6). It is one of the least invasive methods of estimating human mainstream smoke intake assuming the smoker inhales the majority of the smoke drawn into the mouth. The advantage of this approach is that smokers can use their own product in a natural environment with only nominal deviation from their normal behavior by saving the discarded filters.

In the filter analysis approach, a smoking machine is used to smoke cigarettes using a range of defined smoking regimens (puff volume, puff duration, and puff interval) which encompass a wide range of mainstream smoke yields to span most human puffing characteristics. Mainstream smoke total particulate matter (TPM) collected on CFPs and the corresponding cigarette filters are analyzed for nicotine or other smoke constituents allowing relations to be established between chemicals retained in the cigarette filter and on the CFP. Subsequent analysis of cigarette filters collected from smokers allows estimates to be made of mouth-level intake, which smokers received when they consumed those cigarettes. After complications related to differences in filtration efficiency and flow rate were solved by analyzing only the mouth-end portion (downstream from the filter ventilation zone), this method became more widely applicable (6). Published reports indicate strong correlations

between mouth-level nicotine exposure and nicotine metabolite levels in urine and saliva in smokers, suggesting that the measure of smoke exposure using filter analysis are indeed meaningful and robust (4, 7).

Select chemicals have been measured in discarded cigarette filters including nicotine, tobacco-specific nitro-samines (TSNA), and solanesol (5, 8). Solanesol, a non-volatile, stable, and tobacco-specific compound, can serve as a proxy for other chemicals in smoke. Published results indicate excellent linear correlation between cigarette filter solanesol levels and mainstream smoke nicotine or TSNAs levels (9). However, our experiments indicated that the solanesol to BaP relation was not a perfect linear fit. We report here the development of a new method for estimating mouth-level BaP intake by filter analysis. Using this method, we are able to establish a better correlation between BaP in mainstream smoke yield and discarded cigarette filter butts. This method provides a better means for estimating BaP intake and allows an examination of smoking behavior in a naturalistic environment on a cigarette by cigarette basis.

Methods

Standards, reagents, and materials

BaP standard was purchased from Cambridge Isotope Laboratories. Acetonitrile, acetone, and cyclohexane (HPLC grade) were purchased from Sigma. CFPs (44-mm glass fiber filter pad) were obtained from Whatman. Blank cellulose acetate cigarette filters were obtained from Filtrona. Reference cigarettes (2R4F) were from the University of Kentucky (Lexington, KY). Commercial cigarettes were purchased from various retail sources in Atlanta, GA. BaP standard solution (1 μg/mL) was prepared in acetonitrile. For the calibration curves, appropriate volumes of the BaP standard solution were spiked on a set of 7 blank CFPs or seven 1-cm blank cigarette filters (1-cm butt). Each CFP or 1-cm butt was then subjected to the same preparation procedure used for smoked samples.

Smoking regimens and sample collection

Cigarettes and CFPs were conditioned at 22° C and 60% relative humidity for at least 24 hours before smoking. Using a Cerulean ASM500 16-port smoking machine, cigarettes were smoked to the designated puff number (Table 1) or to the length of the filter overwrap plus 3 mm. Mainstream smoke TPM generated under ISO smoking conditions (60-second puff interval, 2-second puff duration, 35-mL puff volume, and no ventilation blocking) or Canadian intense smoking conditions (30-second puff interval, 2-second puff duration, 55-mL puff volume, and 100% ventilation blocking) was collected on individual CFPs. Each CFP traps smoke from one cigarette for each individual sample. After smoking, CFPs and cigarette filters were manually removed and subsequently analyzed.

Sample preparation

Smoke sample preparation from CFPs used a previously published method (10). This method includes cyclo-hexane extraction and solid-phase extraction clean up. These procedures were similar to ones used for cigarette filters except for one additional step: a portion of the cigarette filter (1-cm butt) was removed from the mouth-end, stripped of

wrapping paper, and dissolved in 1-mL acetone. The resulting 1-cm butt slurry solution was then treated with 11-mL cyclohexane and subjected to the same solid-phase extraction clean up as the CFPs.

High-performance liquid chromatography with postcolumn fluorescence derivatization analysis

An Agilent 1200 liquid chromatography coupled with a fluorescence detector (Agilent Technologies) was used to analyze all samples. Samples were injected into a Thermo Hypersil Green PAH column (2.1 × 100 mm inner diameter, 3-μm particle size; Thermo Electron Corporation). The column was equilibrated and run with 100% aceto-nitrile. The flow rate was 250 μL/min, and the run time was 7 minutes. Detection of BaP was achieved by fluorescence at the optimal excitation wavelength of 365 nm and emission wavelength of 415 nm. A second pair of excitation and emission wavelengths (266 and 415 nm, respectively) was also monitored to improve specificity.

Data analysis

ChemStation software (Agilent Technologies) was used to process peak areas. The BaP peak in the chromatogram was automatically selected and integrated. The peak integrations were manually inspected for errors and if necessary, reintegrated. Peak areas integrated under 2 sets of excitation/emission wavelengths (365/415 and 266/415 nm) were used for quantitation and qualification, respectively.

Method validation

Two sets of standards (2, 5, 10, 25, and 50 ng for CFP; 0.5, 1, 2, 5, 10, and 25 ng for cigarette filter) were measured under 2 conditions as follows: (i) directly injected as neat standards and (ii) spiked on CFPs or on 1-cm clean butts and injected after sample preparation to obtain the method recovery rate. The CFP or 1-cm butt recovery was calculated as the ratio of BaP response factors measured after sample preparation over the response factor measured from neat standard.

The detection limit [limit of detection (LOD)] for BaP was estimated from calibration curves as 3 times the SD at the lowest standard concentration.

The accuracy of the method was assessed by spiking known amounts of BaP onto CFPs or 1-cm butts containing TPM collected from smoking 2R4F cigarettes. BaP was spiked at 2 concentrations: one at half the amount in 2R4F cigarette TPM collected on CFP or 1-cm butt, and the other at double the amount in 2R4F. Accuracy was calculated as the mean of the experimentally determined concentration spiked from replicate analyses divided by the nominal concentration. The precision of the method was determined by calculating the relative SDs of 5 replicate measurements.

Filter stability test

Under ISO and Canadian intense (CAN) smoking conditions, a series of 2R4F cigarettes was smoked. Following smoking, cigarette filters were placed in 2-mL Cryovials with screw

tops and stored for up to 3 weeks at room temperature (22° C). These filters were analyzed after 1, 3, 7, 14, and 21 days and compared with ones prepared fresh.

Cigarette physical properties

A Cerulean C2 instrument) was used to measure cigarette length, weight, and filter ventilation. Filter length was measured manually. At least 3 measurements were taken for each parameter, and the average was recorded.

Results

Method development and validation

As a cost saving approach over the previously published high-performance liquid chromatography (HPLC) tandem mass spectrometry method (10), BaP was measured with HPLC coupled with fluorescence detection. The new method has sufficient sensitivity and selectivity for measuring BaP from CFPs and from fresh 1-cm butts. However, with increasing storage time, especially at room temperature, BaP extraction efficiency (recovery rate) from 1-cm butts declines significantly. To improve the extraction efficiency from butts, the procedure was modified by partially dissolving the cigarette butt with acetone. The revised method for both matrices (CFP or 1-cm butt) was validated (Table 2). The estimated method LOD was 0.2 ng for both matrices. The method recovery rates were similar between the 2 matrices and close to previously published data (10). The mean accuracies were 105% for CFP and 96% for cigarette butt. Precisions relative SD were all less than 5% for both matrices.

Filter stability

To assess whether postsmoking storage conditions will affect BaP extraction efficiency, cigarette filters were collected in Cryovials after machine smoking and stored at room temperature for periods of up to 3 weeks. During the 3 weeks storage time, no significant changes were observed at different check points (Fig. 1). Similar results were obtained from both ISO and CAN smoking regimens (Fig. 1).

Calibration smoking

The method was applied to machine-smoked research cigarette 2R4F using a range of smoking parameters (Table 1). These parameters span a wide range of "tar" and nicotine yields that should cover most human smoking parameters. To provide the corresponding calibration equations, BaP levels in mainstream smoke and in the 1-cm butt were subjected to linear regression analysis (Fig. 2). The regression analysis was also tested for proof of concept validation in 3 commercial brands, Marlboro Red (Philip Morris), Newport King (Lorillard), and Camel Silver (R.J. Reynolds). These 3 brands of cigarettes have similar circumference, cigarette length, and tobacco weight; however, they have moderate differences in filter length and dramatic variations in ventilation percentages (0%–55%), which deliver a wide range of "tar" and nicotine levels under machine smoking. Despite these variations, similar linearity was observed in all 3 brands. Brand-specific slopes ranged from 2.7 to 4.3, and the squares of the correlation coefficients (R^2) ranged from 0.93 to 0.98 (Table 3).

Discussion

To estimate smokers' BaP exposure, an amenable and noninvasive approach is measuring mouth-level intake. This approach can be achieved by either duplication of smoking behavior using topography devices or filter analysis of human discarded cigarette filters. Collecting discarded cigarette filters provides a means to observe behavior in a more natural setting for smokers. Smokers' behavior will not be perturbed as it is with topography devices (laboratory setting or cigarette holders). For this reason, along with cost-effectiveness, filter analysis has several advantages for estimating mouth-level intake of BaP and other smoke constituents.

In filter analysis, we found that direct measurement of BaP in cigarette filters provides a better linear relation with mainstream smoke levels than solanesol, although solanesol does serve as an excellent predictor for measuring chemicals in cigarette smoke including nicotine and TSNAs (9). Unlike solanesol and the majority of nicotine and TSNAs trapped in cigarette filters which are transferred from tobacco to smoke, BaP in smoke is a product of various combustion processes.

While testing the potential of measuring BaP trapped in cigarette filters and for recovery comparisons, we evaluated both whole filter and partial filter sections. BaP trapped in whole filters exhibit good linear relation with one in smoke trapped by CFPs only if a single smoking regimen is used. However, no single regimen matches individual smokers' smoking behavior. This observation is especially noticeable for highly ventilated cigarette brands because air drawn through the vents can provide a wide variation in mainstream smoke (5). St. Charles and colleagues have suggested that for most filters, analyses should be conducted on the 1-cm mouth-end portion of filter (downstream of the ventilation zones). This portion's filtration efficiency is relatively constant over a wide range of smoking behaviors (6). Our pilot study on extracting BaP from 1-cm mouth-end butts correlates well with the amount exiting from the filter and captured by CFPs (11). The study also suggests that precise length cutting of each filter tip is crucial to the result. Therefore, we used a custom-designed cigarette filter cutter to cut cigarette filters in batch mode and measured them to the nearest 0.1 mm.

The initial solvent chosen for extracting BaP from cigarette butts was cyclohexane, the same as the one used for extraction from the CFPs. However, we observed that BaP filter extraction efficiency significantly declines with increasing storage time. Time course filter stability experiments indicate reduced extraction of BaP from the filter even after overnight storing at room temperature (11). A similar phenomenon was also observed on nicotine filter extraction by isopropyl alcohol (6). Although BaP extraction efficiency remains stable at −20°C, it is not practical to ask smokers to save their cigarette butts in the freezer. We improved the method by treating butts with acetone first, followed by cyclohexane extraction. The modified method works very well with stability tests (Fig. 1).

A relation between the amount of BaP deposited on the butts and the amount exiting from the filter is determined by machine smoking of individual brands of cigarettes over a variety of smoking parameters. These parameters span a range of puff volumes and intervals to give

cigarette yields that cover those expected from human smoked cigarettes. A linear regression analysis of the cigarette yield of BaP as a function of the amount per butt is used to determine a brand specific calibration equation. The calibration equation can then be applied to estimate human mouth-level intake by measuring the amount of BaP from human smoking cigarette butts and comparing that value against those in the calibration equation. In addition to 2R4F research cigarettes, we chose 3 brands of commercial cigarettes. These 4 brands of cigarettes have different ventilation percentages, which cover a wide range of "tar" and nicotine deliveries. In each brand tested, the amount of BaP from the cigarette butt deposit and the amount of expected mouth-end intake (captured by CFP) shows strong linear relation, with R^2 greater than 0.93 for all cases. The reproducibility, linearity, and R^2 values provide strong evidence that this method is more than adequate to estimate mouth-level intake over a wide range of smoking conditions.

The commercial cigarettes examined have different design features. They all have the same type of filter (cellulose acetate) and similar circumference; modest differences in filter length, cigarette length, and tobacco weight; and dramatically different tip ventilations. All these factors contribute to brand-specific calibrations in the linear regression model generated for each brand (Table 3). We are currently testing more cigarette brands and varieties to investigate which factors have the largest influence on brand specific linearity and response (slope). The study by Hammond and colleagues showed that the Canadian intense smoke regimen maximum delivery can be closer to the mean delivery other than a true maximum in actual smokers' behavior (12). Therefore, we plan to include higher puff volume and/or more frequent puff in current and future studies.

We also observed that despite physical property differences among 4 types of cigarette tested, their linear responses (slopes) only span a narrow range (2.7–4.3). When we combined all calibration data points from 4 cigarette brands, we found a linear regression with R^2 of 0.88 and response of 2.8. Although not as good as individual brand calibration, combined calibration serves as a good indication of mouth-end intake. This feature can be practically useful in certain field study when cigarette butts are obtained without specific brand identities. The data collected to date suggest our method provides an improved means to estimate mouth-level BaP intake using filter analysis. This method is noninvasive and relatively inexpensive. We believe it could have utility and many applications as a tool for examining smoking behavior and exposure-related research studies.

Acknowledgments

The findings and conclusions in this report are those of the author(s) and do not necessarily represent the views of the Centers for Disease Control and Prevention/the Agency for Toxic Substances and Disease Registry. Use of trade names and commercial sources is for identification only and does not imply endorsement by the U.S. Department of Health and Human Services. No potential conflicts of interest were disclosed.

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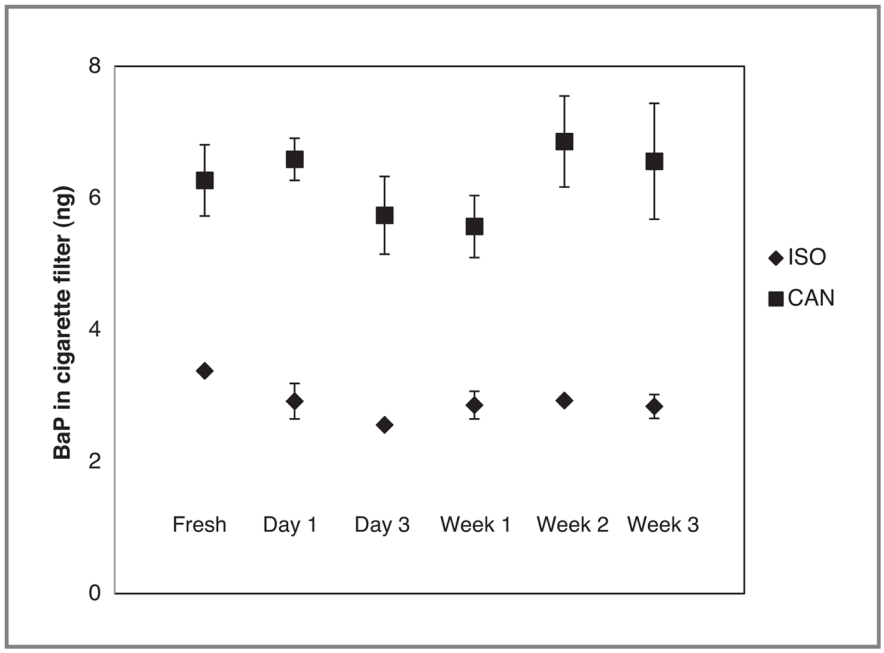

Figure 1.
BaP in 2R4F cigarette filter stability experiment. Each data point is an average of 2 observations.

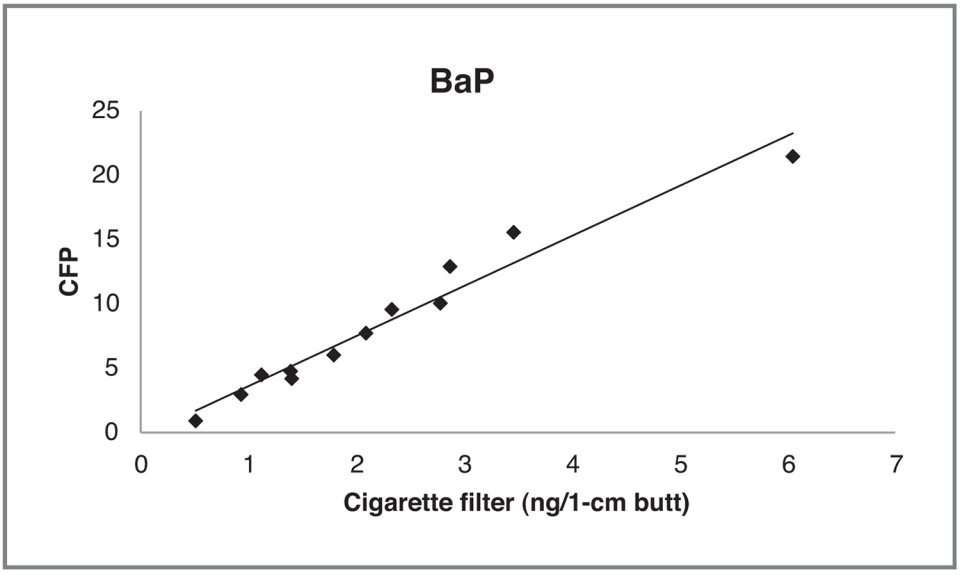

Figure 2.
Relation of BaP between smoke delivery (trapped by CFP) and figlter deposit (1-cm butt) in research cigarette 2R4F.

Table 1

Calibration smoking regimen selection

Regimen	Interval,s	Volume,mL	Puffs or smoked length	Filter vent holes
ISO based	60	35	3	Open
			4	
			5	
			6	
			7	
			T + 3	
CAN based	30	55	4	Closed
			5	
			6	
			7	
			8	
			T + 3	

NOTE: T + 3 = tipping + 3 mm.

Table 2

Method validation parameters

Matrix	Spiking level, ng	Accuracy, %	Precision, %	Recovery, %	LOD, ng
CFP	4	112	4.1	54.9	0.2
	16	98	3.4		
1-cm Butt	1	92	4.0	55.9	
	4	99	2.4		

Table 3

Calibration smoking linearity parameters and cigarette physical properties

Brand	Slope	*y*-Intercept	R^2	Vent tip, %	Circumference,mm	Filter,mm	Cigarette length, mm	Tobacco, g
Marlboro Red	4.3	−0.4	0.97	11.0	24.7	19	79	0.67
2R4F	3.9	−0.3	0.96	32.1	24.7	27	84	0.79
Newport King	3.2	0.8	0.93	0.2	24.8	21	80	0.67
Camel Silver	2.7	−0.1	0.98	54.7	24.4	27	83	0.65